03-10-00

A

Practitioner's Docket No. 49122 **PATENT**

Preliminary Classification:
Proposed Class:
Subclass:
NOTE: *"All applicants are requested to include a preliminary classification on newly filed patent applications. The preliminary classification, preferably class and subclass designations, should be identified in the upper right-hand corner of the letter of transmittal accompanying the application papers, for example 'Proposed Class 2, subclass 129.'" M.P.E.P. § 601, 7th ed.*

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

NEW APPLICATION TRANSMITTAL

Transmitted herewith for filing is the patent application of
Inventor(s): Ståle Petter Lyngstadaas, Lars Hammarström, Stina Gestrelius

WARNING: *37 C.F.R. § 1.41(a)(1) points out:*

"(a) A patent is applied for in the name or names of the actual inventor or inventors.

"(1) The inventorship of a nonprovisional application is that inventorship set forth in the oath or declaration as prescribed by § 1.63, except as provided for in § 1.53(d)(4) and § 1.63(d). If an oath or declaration as prescribed by § 1.63 is not filed during the pendency of a nonprovisional application, the inventorship is that inventorship set forth in the application papers filed pursuant to § 1.53(b), unless a petition under this paragraph accompanied by the fee set forth in § 1.17(i) is filed supplying or changing the name or names of the inventor or inventors."

For (title): MATRIX PROTEIN COMPOSITIONS FOR INDUCTION OF APOPTOSIS

CERTIFICATION UNDER 37 C.F.R. § 1.10*
(Express Mail label number is mandatory.)
(Express Mail certification is optional.)

I hereby certify that this New Application Transmittal and the documents referred to as attached therein are being deposited with the United States Postal Service on this date March 9, 2000, in an envelope as "Express Mail Post Office to Addressee," mailing Label Number TB553893461US, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Peter F. Corless
(type or print name of person mailing paper)

Signature of person mailing paper

WARNING: *Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R. § 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.*

***WARNING:** Each paper or fee filed by "Express Mail" **must** have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. § 1.10(b).*
*"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will **not** be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 56,439, at 56,442.*

1. Type of Application

This new application is for a(n)

(check one applicable item below)

☒ Original (nonprovisional)

☐ Design

☐ Plant

WARNING: ***Do not*** *use this transmittal for a completion in the U.S. of an International Application under 35 U.S.C. § 371(c)(4), unless the International Application is being filed as a divisional, continuation or continuation-in-part application.*

WARNING: ***Do not*** *use this transmittal for the filing of a provisional application.*

NOTE: If one of the following 3 items apply, then complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF A PRIOR U.S. APPLICATION CLAIMED and a NOTIFICATION IN PARENT APPLICATION OF THE FILING OF THIS CONTINUATION APPLICATION.

☐ Divisional.

☐ Continuation.

☐ Continuation-in-part (C-I-P).

2. Benefit of Prior U.S. Application(s) (35 U.S.C. §§ 119(e), 120, or 121)

NOTE: A nonprovisional application may claim an invention disclosed in one or more prior filed copending nonprovisional applications or copending international applications designating the United States of America. In order for a nonprovisional application to claim the benefit of a prior filed copending nonprovisional application or copending international application designating the United States of America, each prior application must name as an inventor at least one inventor named in the later filed nonprovisional application and disclose the named inventor's invention claimed in at least one claim of the later filed nonprovisional application in the manner provided by the first paragraph of 35 U.S.C. § 112. Each prior application must also be:

(i) An international application entitled to a filing date in accordance with PCT Article 11 and designating the United States of America; or

(ii) Complete as set forth in § 1.51(b); or

(iii) Entitled to a filing date as set forth in § 1.53(b) or § 1.53(d) and include the basic filing fee set forth in § 1.16; or

(iv) Entitled to a filing date as set forth in § 1.53(b) and have paid therein the processing and retention fee set forth in § 1.21(l) within the time period set forth in § 1.53(f).

37 C.F.R. § 1.78(a)(1).

NOTE: If the new application being transmitted is a divisional, continuation or a continuation-in-part of a parent case, or where the parent case is an International Application which designated the U.S., or benefit of a prior provisional application is claimed, then check the following item and complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

WARNING: *If an application claims the benefit of the filing date of an earlier filed application under 35 U.S.C. §§ 120, 121 or 365(c), the 20-year term of that application will be based upon the filing date of the earliest U.S. application that the application makes reference to under 35 U.S.C. §§ 120, 121 or 365(c). (35 U.S.C. § 154(a)(2) does not take into account, for the determination of the patent term, any application on which priority is claimed under 35 U.S.C. §§ 119, 365(a) or 365(b).) For a c-i-p application, applicant should review whether any claim in the patent that will issue is supported by an earlier application and, if not, the applicant should consider canceling the reference to the earlier filed application. The term of a patent is not based on a claim-by-claim approach. See Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,205.*

WARNING: *When the last day of pendency of a provisional application falls on a Saturday, Sunday, or Federal holiday within the District of Columbia, any nonprovisional application claiming benefit of the provisional application* ***must*** *be filed prior to the Saturday, Sunday, or Federal holiday within the District of Columbia. See 37 C.F.R. § 1.78(a)(3).*

☒ The new application being transmitted claims the benefit of prior U.S. application(s). Enclosed are ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

3. Papers Enclosed

A. Required for filing date under 37 C.F.R. § 1.53(b) (Regular) or 37 C.F.R. § 1.153 (Design) Application

26 Pages of specification

3 Pages of claims

3 Sheets of drawing

WARNING: ***DO NOT*** *submit original drawings. A high quality copy of the drawings should be supplied when filing a patent application. The drawings that are submitted to the Office must be on strong, white, smooth, and non-shiny paper and meet the standards according to § 1.84. If corrections to the drawings are necessary, they should be made to the original drawing and a high-quality copy of the corrected original drawing then submitted to the Office. Only one copy is required or desired. For comments on proposed then-new 37 C.F.R. § 1.84, see Notice of March 9, 1988 (1990 O.G. 57-62).*

NOTE: "Identifying indicia, if provided, should include the application number or the title of the invention, inventor's name, docket number (if any), and the name and telephone number of a person to call if the Office is unable to match the drawings to the proper application. This information should be placed on the back of each sheet of drawing a minimum distance of 1.5 cm. (5/8 inch) down from the top of the page . . ." 37 C.F.R. § 1.84(c)).

(complete the following, if applicable)

☐ The enclosed drawing(s) are photograph(s), and there is also attached a "PETITION TO ACCEPT PHOTOGRAPH(S) AS DRAWING(S)." 37 C.F.R. § 1.84(b).

☐ formal

☐ informal

B. Other Papers Enclosed

4 Pages of declaration and power of attorney

1 Pages of abstract

____ Other

4. Additional papers enclosed

☐ Amendment to claims

☐ Cancel in this applications claims _____ before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

☐ Add the claims shown on the attached amendment. (Claims added have been numbered consecutively following the highest numbered original claims.)

☐ Preliminary Amendment

☐ Information Disclosure Statement (37 C.F.R. § 1.98)

☐ Form PTO–1449 (PTO/SB/08A and 08B)

☐ Citations

☐ Declaration of Biological Deposit

☐ Submission of "Sequence Listing," computer readable copy and/or amendment pertaining thereto for biotechnology invention containing nucleotide and/or amino acid sequence.

☐ Authorization of Attorney(s) to Accept and Follow Instructions from Representative

☐ Special Comments

☐ Other

5. Declaration or oath (including power of attorney)

NOTE: A newly executed declaration is not required in a continuation or divisional application provided that the prior nonprovisional application contained a declaration as required, the application being filed is by all or fewer than all the inventors named in the prior application, there is no new matter in the application being filed, and a copy of the executed declaration filed in the prior application (showing the signature or an indication thereon that it was signed) is submitted. The copy must be accompanied by a statement requesting deletion of the names of person(s) who are not inventors of the application being filed. If the declaration in the prior application was filed under § 1.47, then a copy of that declaration must be filed accompanied by a copy of the decision granting § 1.47 status or, if a nonsigning person under § 1.47 has subsequently joined in a prior application, then a copy of the subsequently executed declaration must be filed. See 37 C.F.R. §§ 1.63(d)(1)-(3).

NOTE: A declaration filed to complete an application must be executed, identify the specification to which it is directed, identify each inventor by full name including family name and at least one given name, without abbreviation together with any other given name or initial, and the residence, post office address and country or citizenship of each inventor, and state whether the inventor is a sole or joint inventor. 37 C.F.R. § 1.63(a)(1)-(4).

NOTE: "The inventorship of a nonprovisional application is that inventorship set forth in the oath or declaration as prescribed by § 1.62, except as provided for in § 1.53(d)(4) and § 1.63(d). If an oath or declaration as prescribed by § 1.63 is not filed during the pendency of a nonprovisional application, the inventorship is that inventorship set forth in the application papers filed pursuant to § 1.53(b), unless a petition under this paragraph accompanied by the fee set forth in § 1.17(i) is filed supplying or changing the name or names of the inventor or inventors." 37 C.F.R. § 1.41(a)(1).

☒ Enclosed

Executed by

(check **all** *applicable boxes)*

☒ inventor(s).

☐ legal representative of inventor(s). 37 C.F.R. §§ 1.42 or 1.43.

☐ joint inventor or person showing a proprietary interest on behalf of inventor who refused to sign or cannot be reached.

☐ This is the petition required by 37 C.F.R. § 1.47 and the statement required by 37 C.F.R. § 1.47 is also attached. See item 13 below for fee.

☐ Not Enclosed.

NOTE: Where the filing is a completion in the U.S. of an International Application or where the completion of the U.S. application contains subject matter in addition to the International Application, the application may be treated as a continuation or continuation-in-part, as the case may be, utilizing ADDED PAGE FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION CLAIMED.

☐ Application is made by a person authorized under 37 C.F.R. § 1.41(c) on behalf of *all* the above named inventor(s).

(The declaration or oath, along with the surcharge required by 37 C.F.R. § 1.16(e) can be filed subsequently).

☐ Showing that the filing is authorized.
(not required unless called into question. 37 C.F.R. § 1.41(d))

6. Inventorship Statement

WARNING: *If the named inventors are each not the inventors of all the claims an explanation, including the ownership of the various claims at the time the last claimed invention was made, should be submitted.*

The inventorship for all the claims in this application are:

☐ The same.

or

☐ Not the same. An explanation, including the ownership of the various claims at the time the last claimed invention was made,

☐ is submitted.

☐ will be submitted.

7. Language

NOTE: An application including a signed oath or declaration may be filed in a language other than English. An English translation of the non-English language application and the processing fee of $130.00 required by 37 C.F.R. § 1.17(k) is required to be filed with the application, or within such time as may be set by the Office. 37 C.F.R. § 1.52(d).

☒ English

☐ Non-English

☐ The attached translation includes a statement that the translation is accurate. 37 C.F.R. § 1.52(d).

8. Assignment

☒ An assignment of the invention to Biora BioEx AB of Malmo, Sweden

☒ is attached. A separate ☐ "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or ☒ FORM PTO 1595 is also attached.

☐ will follow.

NOTE: "If an assignment is submitted with a new application, send two separate letters-one for the application and one for the assignment." Notice of May 4, 1990 (1114 O.G. 77-78).

WARNING: *A newly executed "CERTIFICATE UNDER 37 C.F.R. § 3.73(b)" must be filed when a continuation-in-part application is filed by an assignee. Notice of April 30, 1993, 1150 O.G. 62-64.*

9. Certified Copy

Certified copy(ies) of application(s)

Denmark	PA 1999 00336	March 10, 1999
Country	Appln. No.	Filed
Country	Appln. No.	Filed
Country	Appln. No.	Filed

from which priority is claimed

☒ is (are) attached.

☐ will follow.

NOTE: The foreign application forming the basis for the claim for priority must be referred to in the oath or declaration. 37 C.F.R. § 1.55(a) and 1.63.

NOTE: This item is for any foreign priority for which the application being filed directly relates. If any parent U.S. application or International Application from which this application claims benefit under 35 U.S.C. § 120 is itself entitled to priority from a prior foreign application, then complete item 18 on the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

10. Fee Calculation (37 C.F.R. § 1.16)

A. ☒ Regular application

CLAIMS AS FILED					
Number filed		Number Extra	Rate	Basic Fee 37 C.F.R. § 1.16(a) $690.00	
Total Claims (37 C.F.R. § 1.16(c))	32 − 20 =	12	×	$ 18.00	216.00
Independent Claims (37 C.F.R. § 1.16(b))	3 − 3 =		×	$ 78.00	
Multiple dependent claim(s), if any (37 C.F.R. § 1.16(d))			+	$260.00	260.00

☐ Amendment cancelling extra claims is enclosed.

☐ Amendment deleting multiple-dependencies is enclosed.

☐ Fee for extra claims is not being paid at this time.

NOTE: If the fees for extra claims are not paid on filing they must be paid or the claims cancelled by amendment, prior to the expiration of the time period set for response by the Patent and Trademark Office in any notice of fee deficiency. 37 C.F.R. § 1.16(d).

Filing Fee Calculation $ 1,166.00

B. ☐ Design application ($310.00—37 C.F.R. § 1.16(f))

Filing Fee Calculation $_____

C. ☐ Plant application
($480.00—37 C.F.R. § 1.16(g))

Filing fee calculation $_____

11. Small Entity Statement(s)

☐ Statement(s) that this is a filing by a small entity under 37 C.F.R. § 1.9 and 1.27 is (are) attached.

WARNING: *"Status as a small entity must be specifically established in each application or patent in which the status is available and desired. Status as a small entity in one application or patent does not affect any other application or patent, including applications or patents which are directly or indirectly dependent upon the application or patent in which the status has been established. The refiling of an application under § 1.53 as a continuation, division, or continuation-in-part (including a continued prosecution application under § 1.53(d)), or the filing of a reissue application requires a new determination as to continued entitlement to small entity status for the continuing or reissue application. A nonprovisional application claiming benefit under 35 U.S.C. § 119(e), 120, 121, or 365(c) of a prior application, or a reissue application may rely on a statement filed in the prior application or in the patent if the nonprovisional application or the reissue application includes a reference to the statement in the prior application or in the patent or includes a copy of the statement in the prior application or in the patent and status as a small entity is still proper and desired. The payment of the small entity basic statutory filing fee will be treated as such a reference for purposes of this section." 37 C.F.R. § 1.28(a)(2).*

WARNING: *"Small entity status must not be established when the person or persons signing the . . . statement can **unequivocally** make the required self-certification." M.P.E.P., § 509.03, 6th ed., rev. 2, July 1996 (emphasis added).*

(complete the following, if applicable)

☐ Status as a small entity was claimed in prior application

_____ / _____, filed on _____, from which benefit is being claimed for this application under:

35 U.S.C. § ☐ 119(e),
☐ 120,
☐ 121,
☐ 365(c),

and which status as a small entity is still proper and desired.

☐ A copy of the statement in the prior application is included.

Filing Fee Calculation (50% of **A, B** or **C** above)

$_____

NOTE: Any excess of the full fee paid will be refunded if small entitiy status is established and a refund request are filed within 2 months of the date of timely payment of a full fee. The two-month period is not extendable under § 1.136. 37 C.F.R. § 1.28(a).

12. Request for International-Type Search (37 C.F.R. § 1.104(d))

(complete, if applicable)

☐ Please prepare an international-type search report for this application at the time when national examination on the merits takes place.

13. Fee Payment Being Made at This Time

☐ Not Enclosed

☐ No filing fee is to be paid at this time.
(This and the surcharge required by 37 C.F.R. § 1.16(e) can be paid subsequently.)

☒ Enclosed

☒ Filing fee	$ 1,166.00
☒ Recording assignment ($40.00; 37 C.F.R. § 1.21(h)) (See attached "COVER SHEET FOR ASSIGNMENT ACCOMPANYING NEW APPLICATION".)	$ 40.00
☐ Petition fee for filing by other than all the inventors or person on behalf of the inventor where inventor refused to sign or cannot be reached ($130.00; 37 C.F.R. §§ 1.47 and 1.17(i))	$
☐ For processing an application with a specification in a non-English language ($130.00; 37 C.F.R. §§ 1.52(d) and 1.17(k))	$
☐ Processing and retention fee ($130.00; 37 C.F.R. §§ 1.53(d) and 1.21(l))	$
☐ Fee for international-type search report ($40.00; 37 C.F.R. § 1.21(e))	$

NOTE: 37 C.F.R. § 1.21(l) establishes a fee for processing and retaining any application that is abandoned for failing to complete the application pursuant to 37 C.F.R. § 1.53(f) and this, as well as the changes to 37 C.F.R. §§ 1.53 and 1.78(a)(1), indicate that in order to obtain the benefit of a prior U.S. application, either the basic filing fee must be paid, or the processing and retention fee of § 1.21(l) must be paid, within 1 year from notification under § 53(f).

Total fees enclosed $ 1,206.00

14. Method of Payment of Fees

☒ Check in the amount of $ 1,206.00

☐ Charge Account No. _____ in the amount of $_____

A duplicate of this transmittal is attached.

NOTE: Fees should be itemized in such a manner that it is clear for which purpose the fees are paid. 37 C.F.R. § 1.22(b).

15. Authorization to Charge Additional Fees

WARNING: *If no fees are to be paid on filing, the following items should not be completed.*

WARNING: *Accurately count claims, especially multiple dependent claims, to avoid unexpected high charges, if extra claim charges are authorized.*

☒ The Commissioner is hereby authorized to charge the following additional fees by this paper and during the entire pendency of this application to Account No. 04-1105 :

☒ 37 C.F.R. § 1.16(a), (f) or (g) (filing fees)

☒ 37 C.F.R. § 1.16(b), (c) and (d) (presentation of extra claims)

NOTE: Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 C.F.R. § 1.16(d)), it might be best not to authorize the PTO to charge additional claim fees, except possibly when dealing with amendments after final action.

☒ 37 C.F.R. § 1.16(e) (surcharge for filing the basic filing fee and/or declaration on a date later than the filing date of the application)

☒ 37 C.F.R. § 1.17(a)(1)-(5) (extension fees pursuant to § 1.136(a)).

☒ 37 C.F.R. § 1.17 (application processing fees)

NOTE: ". . .A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1.17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 C.F.R. § 1.136(a)(3).

☐ 37 C.F.R. § 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. § 1.311(b))

NOTE: Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 C.F.R. § 1.311(b).

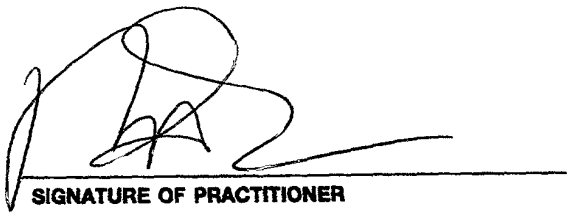
NOTE: 37 C.F.R. § 1.28(b) requires "Notification of any change in status resulting in loss of entitlement to small entity status must be filed in the application . . . prior to paying, or at the time of paying, . . . the issue fee. . . " From the wording of 37 C.F.R. § 1.28(b), (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.

16. Instructions as to Overpayment

NOTE: ". . . Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 C.F.R. § 1.26(a).

☒ Credit Account No. 04-1105

☐ Refund



SIGNATURE OF PRACTITIONER

Reg. No. 33,860

Peter F. Corless

(type or print name of attorney)

Tel. No. (617) 523-3400

Dike, Bronstein, Roberts & Cushman, LLP

130 Water Street

P.O. Address

Customer No.

Boston, MA 02109

☒ **Incorporation by reference of added pages**

(check the following item if the application in this transmittal claims the benefit of prior U.S. application(s) (including an international application entering the U.S. stage as a continuation, divisional or C-I-P application) and complete and attach the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED)

☒ Plus Added Pages for New Application Transmittal Where Benefit of Prior U.S. Application(s) Claimed

Number of pages added 5

☐ Plus Added Pages for Papers Referred to in Item 4 Above

Number of pages added ______

☐ Plus added pages deleting names of inventor(s) named in prior application(s) who is/are no longer inventor(s) of the subject matter claimed in this application.

Number of pages added ______

☒ Plus "Assignment Cover Letter Accompanying New Application"

Number of pages added 3

☐ **Statement Where No Further Pages Added**

(if no further pages form a part of this Transmittal, then end this Transmittal with this page and check the following item)

☐ This transmittal ends with this page.

Practitioner's Docket No. 49122 **PATENT**

ADDED PAGES FOR APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED

NOTE: See 37 C.F.R. § 1.78.

17. Relate Back

WARNING: *If an application claims the benefit of the filing date of an earlier filed application under 35 U.S.C. §§ 120, 121 or 365(c), the 20-year term of that application will be based upon the filing date of the earliest U.S. application that the application makes reference to under 35 U.S.C. §§ 120, 121 or 365(c). (35 U.S.C. § 154(a)(2) does not take into account, for the determination of the patent term, any application on which priority is claimed under 35 U.S.C. §§ 119, 365(a) or 365(b).) For a c-i-p application, applicant should review whether any claim in the patent that will issue is supported by an earlier application and, if not, the applicant should consider canceling the reference to the earlier filed application. The term of a patent is not based on a claim-by-claim approach. See Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,205.*

(complete the following, if applicable)

☒ Amend the specification by inserting, before the first line, the following sentence:

A. 35 U.S.C. § 119(e)

NOTE: "Any nonprovisional application claiming the benefit of one or more prior filed copending provisional applications must contain or be amended to contain in the first sentence of the specification following the title a reference to each such prior provisional application, identifying it as a provisional application, and including the provisional application number (consisting of series code and serial number)." 37 C.F.R. § 1.78(a)(4).

☒ "This application claims the benefit of U.S. Provisional Application(s) No(s).:

APPLICATION NO(S).:	FILING DATE
60 / 134,813	May 19, 1999 "
/	"
/	"

B. 35 U.S.C. §§ 120, 121 and 365(c)

NOTE: "Except for a continued prosecution application filed under § 1.53(d), any nonprovisional application claiming the benefit of one or more prior filed copending nonprovisional applications or international applications designating the United States of America must contain or be amended to contain in the first sentence of the specification following the title a reference to each such prior application, identifying it by application number (consisting of the series code and serial number) or international application number and international filing date and indicating the relationship of the applications. . . . Cross-references to other related applications may be made when appropriate." (See § 1.14(a)). 37 C.F.R. § 1.78(a)(2).

☐ "This application is a

- ☐ continuation
- ☐ continuation-in-part
- ☐ divisional

of copending application(s)

☐ application number 0 /_____ filed on _____"

☐ International Application _____ filed on _____ and which designated the U.S."

NOTE: The proper reference to a prior filed PCT application that entered the U.S. national phase is the U.S. serial number and the filing date of the PCT application that designated the U.S.

NOTE: (1) Where the application being transmitted adds subject matter to the International Application, then the filing can be as a continuation-in-part or (2) if it is desired to do so for other reasons then the filing can be as a continuation.

NOTE: The deadline for entering the national phase in the U.S. for an international application was clarified in the Notice of April 28, 1987 (1079 O.G. 32 to 46) as follows:

"The Patent and Trademark Office considers the International application to be pending until the 22nd month from the priority date if the United States has been designated and no Demand for International Preliminary Examination has been filed prior to the expiration of the 19th month from the priority date and until the 32nd month from the priority date if a Demand for International Preliminary Examination which elected the United States of America has been filed prior to the expiration of the 19th month from the priority date, provided that a copy of the international application has been communicated to the Patent and Trademark Office within the 20 or 30 month period respectively. If a copy of the international application has not been communicated to the Patent and Trademark Office within the 20 or 30 month period respectively, the international application becomes abandoned as to the United States 20 or 30 months from the priority date respectivley. These periods have been placed in the rules as paragraph (h) of § 1.494 and paragraph (i) of § 1.495. A continuing application under 35 U.S.C. 365(c) and 120 may be filed anytime during the pendency of the international application."

☐ "The nonprovisional application designated above, namely application _____ / _____, filed _____, claims the benefit of U.S. Provisional Application(s) No(s).:

APPLICATION NO(S).:	**FILING DATE**
_____ /_____	_____"
_____ /_____	_____"
_____ /_____	_____"

☐ Where more than one reference is made above, please combine all references into one sentence.

18. Relate Back—35 U.S.C. § 119 Priority Claim for Prior Application

The prior U.S. application(s), including any prior International Application designating the U.S., identified above in item 17B, in turn itself claim(s) foreign priority(ies) as follows:

Country	Appln. no.	Filed on
Denmark	PA 1999 00336	March 10, 1999

The certified copy(ies) has (have)

☐ been filed on _____, in prior application 0 /_____, which was filed on _____.

☒ is (are) attached.

WARNING: *The certified copy of the priority application that may have been communicated to the PTO by the International Bureau may* ***not*** *be relied on without any need to file a certified copy of the priority application* ***in the continuing application.*** *This is so because the certified copy of the priority application communicated by the International Bureau is placed in a folder and is not assigned a U.S. serial number unless the national stage is entered. Such folders are disposed of if the national stage is not entered. Therefore, such certified copies may not be available if needed later in the prosecution of a continuing application. An alternative would be to physically remove the priority documents from the folders and transfer them to the continuing application. The resources required to request transfer, retrieve the folders, make suitable record notations, transfer the certified copies, enter and make a record of such copies in the Continuing Application are substantial. Accordingly, the priority documents in folders of international applications that have not entered the national stage may not be relied on. Notice of April 28, 1987 (1079 O.G. 32 to 46).*

19. Maintenance of Copendency of Prior Application

NOTE: The PTO finds it useful if a copy of the petition filed in the prior application extending the term for response is filed with the papers constituting the filing of the continuation application. Notice of November 5, 1985 (1060 O.G. 27).

A. ☐ Extension of time in prior application

(This item ***must*** *be completed and the papers filed* ***in the prior application,*** *if the period set in the prior application has run.)*

☐ A petition, fee and response extends the term in the pending **prior** application until _____

☐ A **copy** of the petition filed in prior application is attached.

B. ☐ Conditional Petition for Extension of Time in Prior Application

(complete this item, if previous item not applicable)

☐ A conditional petition for extension of time is being filed in the **pending prior** application.

☐ A **copy** of the conditional petition filed in the prior application is attached.

20. Further Inventorship Statement Where Benefit of Prior Application(s) Claimed

(complete applicable item (a), (b) and/or (c) below)

(a) ☐ This application discloses and claims only subject matter disclosed in the prior application whose particulars are set out above and the inventor(s) in this application are

- ☐ the same.
- ☐ less than those named in the prior application. It is requested that the following inventor(s) identified for the prior application be deleted:

_____

(type name(s) of inventor(s) to be deleted)

(b) ☐ This application discloses and claims additional disclosure by amendment and a new declaration or oath is being filed. With respect to the prior application, the inventor(s) in this application are

- ☐ the same.
- ☐ the following additional inventor(s) have been added:

_____

(type name(s) of inventor(s) to be added)

(c) The inventorship for all the claims in this application are

- ☐ the same.
- ☐ not the same. An explanation, including the ownership of the various claims at the time the last claimed invention was made
  - ☐ is submitted.
  - ☐ will be submitted.

21. Abandonment of Prior Application *(if applicable)*

☐ Please abandon the prior application at a time while the prior application is pending, or when the petition for extension of time or to revive in that application is granted, and when this application is granted a filing date, so as to make this application copending with said prior application.

NOTE: According to the Notice of May 13, 1983 (103, TMOG 6-7), the filing of a continuation or continuation-in-part application is a proper response with respect to a petition for extension of time or a petition to revive and should include the express abandonment of the prior application conditioned upon the granting of the petition and the granting of a filing date to the continuing application.

22. Petition for Suspension of Prosecution for the Time Necessary to File an Amendment

WARNING: *"The claims of a new application may be finally rejected in the first Office action in those situations where (A) the new application is a continuing application of, or a substitute for, an earlier application, and (B) all the claims of the new application (1) are drawn to the same invention claimed in the earlier application, and (2) would have been properly finally rejected on the grounds of art of record in the next Office action if they had been entered in the earlier application." M.P.E.P., § 706.07(b), 7th ed.*

NOTE: Where it is possible that the claims on file will give rise to a first action final for this continuation application and for some reason an amendment cannot be filed promptly (e.g., experimental data is being gathered) it may be desirable to file a petition for suspension of prosecution for the time necessary.

(check the next item, if applicable)

☐ There is provided herewith a Petition To Suspend Prosecution for the Time Necessary to File An Amendment (New Application Filed Concurrently)

23. Small Entity (37 C.F.R. § 1.28(a))

☐ Applicant has established small entity status by the filing of a statement in parent application /_____ on _____ .

☐ A copy of the statement previously filed is included.

WARNING: *See 37 C.F.R. § 1.28(a).*

WARNING: *"Small entity status must not be established when the person or persons signing the . . . statement can **unequivocally** make the required self-certification." M.P.E.P., § 509.03, 7th ed. (emphasis added).*

24. NOTIFICATION IN PARENT APPLICATION OF THIS FILING

☐ A notification of the filing of this
(check one of the following)

☐ continuation

☐ continuation-in-part

☐ divisional

is being filed in the parent application, from which this application claims priority under 35 U.S.C. § 120.

Docket No. 49122
Express Mail Label No. TB553893461

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
NEW PATENT APPLICATION

TITLE: MATRIX PROTEIN COMPOSITIONS FOR INDUCTION OF APOPTOSIS

INVENTORS: Ståle Petter Lyngstadaas
Lars Hammarström
Stina Gestrelius

ATTORNEY: Peter F. Corless (Reg. No. 33,860)
DIKE, BRONSTEIN, ROBERTS
& CUSHMAN, LLP
130 Water Street
Boston, Massachusetts 02109
Tel: (617) 523-3400
Fax: (617) 523-6440

MATRIX PROTEIN COMPOSITIONS FOR INDUCTION OF APOPTOSIS

FIELD OF INVENTION

The present invention relates to the use of enamel matrix, enamel matrix derivatives and/or enamel matrix proteins or peptides as therapeutic or prophylactic agents for inducing programmed cell death.

BACKGROUND OF THE INVENTION

Enamel matrix proteins such as those present in enamel matrix are best known as precursors of dental enamel. Enamel proteins and enamel matrix derivatives have previously been described in the patent literature to induce hard tissue formation (i.e. enamel formation, cf. US Patent No. 4,672,032 (Slavkin)) or binding between hard tissues (EP-B-0 337 967 and EP-B-0 263 086). In recent animal studies using enamel matrix proteins for regeneration of tooth attachment, it has been observed that the regeneration and healing progresses with minimal signs of epithelial interference. This is in contrast to all other regenerative therapies of the periodontium where epithelial downgrowth from the oral cavity into the lesion is a common complication. To investigate possible restrictive effects of enamel matrix proteins on epithelial cell growth, epithelial cells were cultured in the presence of enamel matrix proteins.

SUMMARY OF THE INVENTION

It has surprisingly been found that epithelial cancer cells (HeLa cells) which are not ameloblasts and which are not found in the periodontal environment and which do not participate in tooth development undergo apoptosis (programmed or induced cell death) when cultured in the presence of enamel matrix, enamel matrix derivatives and/or enamel matrix proteins (in the following collectively termed "active enamel substance"). Accordingly, the present invention relates to the use of a preparation of an active enamel substance for the preparation of a pharmaceutical composition for the induction of apoptosis.

In another aspect, the invention relates to the use of a preparation of an active enamel substance for the preparation of a pharmaceutical composition for the prevention or treatment of malignant or benign neoplasms.

In a further aspect, the invention relates to the use of a preparation of an active enamel substance for the preparation of a pharmaceutical composition for the prevention or treatment of cancer.

In a still further aspect, the invention relates to the use of a preparation of an active enamel substance for the preparation of a pharmaceutical composition for the (selective) induction of apoptosis in neoplastic cells.

In a still further aspect, the present invention relates to method for inducing apoptosis in neoplastic cells, the method comprising applying an effective amount of an active enamel substance at or on neoplastic cells.

Apoptosis (programmed or induced cell death) is involved in the focal elimination of certain cells during normal development and in the turnover of cells in healthy adult tissues. Examples where apoptosis has been found to be involved are in organogenesis during embryonic life, e.g. the separation of digits during limb development, cusp and root formation during tooth development, elimination of worn-out cells in the small intestine, and clonal elimination of lymphocytes that might otherwise react with "self" antigens.

Recent evidence suggests that apoptosis may be equally important in the understanding of carcinogenesis and for developing novel therapies for different neoplastic diseases. Thus, it has been shown that apoptosis is involved in preventing genome instability from developing in cells in which the cell cycle has been perturbed, and it is associated with injurious stimuli such as therapeutic irradiation and cytotoxic drugs used to treat malignant neoplasms.

Apoptosis is a gene-regulated process involving the synthesis of some proteins that promote apoptosis and others that protect against it. The presence of the tumour suppressor gene p53 is responsible for the initiation of apoptosis as a result of cell injury, in particular injury caused by DNA double-stranded breaks. In human cancer treatment, this is extremely important because tumour cells from which p53 is absent do not undergo apoptosis when exposed to ionising radiation. In addition, lack of p53 is likely to result in the survival of cells in which DNA mutations have occurred and thus to increase the risk of development of cancer. Certain cells that have been deprived of growth promoting factors or whose growth have been arrested with cytotoxic drugs are rendered susceptible to apoptosis by expression of the

proto-oncogene c-myc. This gene encodes an essential part of the proliferative machinery of the cell, and deregulation of the expression of this gene is implicated in most neoplasms. Certain gene products protect cells from apoptosis. Examples of such gene products include those of the bcl-2 gene, the proto-oncogene c-abl and the LMP-1 gene of the Epstein-Barr virus.

A number of different pathways for induction of apoptosis have been demonstrated. Gamma irradiation acts directly on the chromosomes by causing DNA strand breaks. Certain hormones such as glucocorticoids induce apoptosis in thymocytes, probably via a glucocorticoid receptor complex. A third induction pathway is via a direct contact with the plasma membrane of the target cells. An example of this is the effect of granzyme B which is released as part of the interaction of cytotoxic T-cells with a target cell.

In epithelial cells, apoptosis has been found to take place when cell adhesion to extracellular matrix by means of fibronectin and other extracellular proteins is blocked. In an immortalised thyroid cell line, it appeared that adhesion, spreading and cytoskeleton organisation were dependent on integrin-fibronectin interaction. It has been shown that cells become apoptotic when peptides containing the RGD (Arg-Gly-Asp) motif inhibit binding of fibronectin to integrin receptors (cf. M. Vitale et al., J. Clin. Endocrinol. Metab. 83 (10), 1998, pp. 3673-3680). Similar findings have been reported for normal cells by, e.g., Z Zhang et al., Proc. Natl. Acad. Sci. USA 92 (13), 1995, pp. 6161-6165; HJ Kim et al., Am. J. Physiol. 271 (2 pt.1), 1996, pp. L277-286; G McGill et al., J. Cell Biol. 138 (4), 1997, pp. 901-911. Inhibition of adhesion resulting in cell death not only happens to normal (non-transformed) cells, but has also been shown for melanoma cells. Thus, MD Mason et al., J. R. Soc. Med. 89 (7), 1996, pp. 393-395, reported that loss of integrin-mediated signalling induced apoptosis in melanoma cells within 3 days of treatment with a peptide containing the RGD motif blocking the alpha v beta 3 integrin. Furthermore, it has been shown that the RDG motif is an integrin-recognition motif, and that peptides containing RDG induce apoptosis by activation of caspase-3 which is an enzyme that participates in a cascade resulting in disassembly of the cell (cf. CD Buckley et al., Nature 397, Feb. 1999, pp. 534-539).

Without wishing to be limited to any particular hypothesis, it is currently believed that the active enamel substance may exert its apoptosis inducing effect either by binding to cell surface receptors in an analogous manner to RDG so as to block the site needed for cellular adhesion to extracellular matrix, or by binding to cells surface structures such as CD44 or integrins, thereby directly activating caspase 3 and triggering the apoptotic pathway described by Buckley et al., supra.

In developing teeth, the enamel is formed by a layer of epithelial cells called ameloblasts. These, in turn, are supported by another layer of epithelial cells providing the ameloblasts with growth factors and nutrients required for their continued existence and function. The ameloblasts synthesize and secrete enamel matrix proteins which, together with mineral and water, consitute the enamel matrix which is an early developmental stage of dental enamel. Enamel matrix proteins are mainly present during the secretory stage of enamel formation. After their initial deposition, they are gradually degraded and then lost as enamel development progresses. Also, during enamel development, apoptosis is observed in epithelial cells close to the enamel matrix. This apoptotic event is preceded by translocation of enamel matrix degradation products from the developing enamel into the enamel organ that is composed of epithelial cells. The active enamel substance observed to be useful in the present invention is composed of a number of proteins and peptides including such degradation products. These observations are also supported by studies of guinea pig molars showing that processing of enamel proteins is linked to the reduction of the number of surrounding epithelial cells (cf. Example 1 below).

DETAILED DESCRIPTION OF THE INVENTION

Apoptosis has attracted considerable interest for the potential treatment of cancers and neoplasms. The present inventors surprisingly observed that when human epithelial cancer cells (HeLa cells) were cultured in the presence of the active enamel substance they underwent apoptosis (vide Example 2 below). By way of comparison, human connective tissue cells (fibroblasts) cultured under similar conditions in the presence of the active enamel substance were stimulated as to growth. Based on these results, the present inventors believe that the active enamel substance may be used for the (selective) induction of apoptosis in neoplastic cells, specifically in the treatment, e.g. topical treatment, of certain types of cancer and benign, semi-malignant (i.e. locally invasive) or malignant neoplasms.

It is currently believed that the active enamel substance may be particularly beneficial for use in the treatment of epithelially derived cancers or neoplasms, as the results currently available appear to show that the active enamel substance exerts its apoptotic effect specifically on epithelial cells. In the use according to the present invention, it is therefore preferred to apply the active enamel substance topically at or on affected tissue comprising a substantial proportion of epithelial cells. In particular, such tissue comprises skin or mucosal tissue. Mucosal tissue which may advantageously be treated with the active enamel substance in accordance with the invention comprises any tissue which presents

a suitable surface available for topical application of the active enamel substance, either naturally or following surgical incision. Examples of such mucosal surfaces are oral, gastrointestinal, respiratory tract (e.g. lung), cervical or abdominal mucosa.

Other tissues comprising a significant proportion of epithelial cells are glandular tissues, e.g. mammary gland, pancreas, liver, thyroid gland, bladder, ovary, prostate, sweat gland, salivary gland or pituitary gland tissue.

It has been found, however, that cancer cells derived from other tissues than epithelial or mucosal tissue or other tissues comprising a significant number of epithelial cells also exhibit a marked increase in apoptosis when treated with the preparation of active enamel substance according to the invention (*vide* Example 3 below). The present invention therefore extends to the use of a preparation of the active enamel substance in the treatment of cancers or neoplasms in tissue such as bone or muscle tissue.

On surgical removal of a tumour, it is important to reduce the risk that tumour cells migrate from the site of surgery to invade another part of the body. In a specific embodiment of the present invention, the active enamel substance is therefore applied at or on a tumour site before, during or after surgical removal of a tumour or neoplastic tissue to substantially reduce the risk of postsurgical metastasis and/or to prevent recurrence of the tumour at the site of surgery. In particular, it is envisaged that the preparation of active enamel substance may be applied for adjuvant cancer therapy, e.g. in conjunction with conventional radiation therapy. It is currently believed that such adjuvant therapy using the active enamel substance may both reduce the risk of tumour cell migration (i.e. metastasis) in accordance with the present findings and contribute to the healing of wounds often resulting from radiation therapy as the active enamel substance has also been found to exhibit wound healing properties (*vide* for instance WO 99/43344).

Apart from the treatment of cancer or neoplastic tissue, it is contemplated that the active enamel substance may be used for the prophylaxis or treatment of warts, in particular warts resulting from viral infection, e.g. papilloma or condyloma.

Enamel matrix, enamel matrix derivatives and enamel matrix proteins

Enamel matrix is a precursor to enamel and may be obtained from any relevant natural source, i.e. a mammal in which teeth are under development. A suitable source is developing teeth from slaughtered animals such as, e.g., calves, pigs or lambs. Another source is for example fish skin.

Enamel matrix can be prepared from developing teeth as described previously (EP-B-0 337 967 and EP-B-0 263 086). The enamel matrix is scraped off and enamel matrix derivatives are prepared, e.g. by extraction with aqueous solution such as a buffer, a dilute acid or base or a water/solvent mixture, followed by size exclusion, desalting or other purification steps, optionally followed by freeze-drying. Enzymes may be deactivated by treatment with heat or solvents, in which case the derivatives may be stored in liquid form without freeze-drying.

In the present context, enamel matrix derivatives are derivatives of enamel matrix which include one or several of enamel matrix proteins or parts of such proteins, produced naturally by alternate splicing or processing, or by either enzymatic or chemical cleavage of a natural length protein, or by synthesis of polypeptides in vitro or in vivo (recombinant DNA methods or cultivation of diploid cells). Enamel matrix protein derivatives also include enamel matrix related polypeptides or proteins. The polypeptides or proteins may be bound to a suitable biodegradable carrier molecule, such as polyamino acids or polysaccharides, or combinations thereof. Furthermore, the term enamel matrix derivatives also encompasses synthetic analogous substances.

Proteins are biological macromolecules constituted by amino acid residues linked together by peptide bonds. Proteins, as linear polymers of amino acids, are also called polypeptides. Typically, proteins have 50-800 amino acid residues and hence have molecular weights in the range of from about 6,000 to about several hundred thousand Daltons or more. Small proteins are called peptides or oligopeptides.

Enamel matrix proteins are proteins which normally are present in enamel matrix, i.e. the precursor for enamel (Ten Cate: Oral Histology, 1994; Robinson: Eur. J. Oral Science, Jan. 1998, 106 Suppl. 1:282-91), or proteins which can be obtained by cleavage of such proteins. In general such proteins have a molecular weight below 120,000 daltons and include amelogenins, non-amelogenins, proline-rich non-amelogenins, amelins (ameloblastin, sheathlin) and tuftelins.

Examples of proteins for use according to the invention are amelogenins, proline-rich non-amelogenins, tuftelin, tuft proteins, serum proteins, salivary proteins, amelin, enamelin, ameloblastin, sheathlin, and derivatives thereof, and mixtures thereof. A preparation containing an active enamel substance for use according to the invention may also contain at least two of the aforementioned proteinaceous substances. A commercial product comprising amelogenins and possibly other enamel matrix proteins is marketed as EMDOGAIN® (Biora AB).

In general, the major proteins of an enamel matrix are known as amelogenins. They constitute about 90% w/w of the matrix proteins. The remaining 10% w/w includes proline-rich non-amelogenins, tuftelin, tuft proteins, serum proteins and at least one salivary protein; however, other proteins may also be present such as, e.g., amelin (ameloblastin, sheathlin) which have been identified in association with enamel matrix. Furthermore, the various proteins may be synthesized and/or processed in several different sizes (i.e. different molecular weights). Thus, the dominating proteins in enamel matrix, amelogenins, have been found to exist in several different sizes which together form supramolecular aggregates. They are markedly hydrophobic substances which under physiologically conditions form aggregates. They may carry or be carriers for other proteins or peptides.

Other protein substances are also contemplated to be suitable for use according to the present invention. Examples include proteins such as proline-rich proteins and polyproline. Other examples of substances which are contemplated to be suitable for use according to the present invention are aggregates of such proteins, of enamel matrix derivatives and/or of enamel matrix proteins as well as metabolites of enamel matrix, enamel matrix derivatives and enamel matrix proteins. The metabolites may be of any size ranging from the size of proteins to that of short peptides.

As mentioned above, the proteins, polypeptides or peptides for use according to the invention typically have a molecular weight of at the most about 120 kDa such as, e.g., at the most 100 kDa, 90 kDa, 80 kDa, 70 kDa or 60 kDa as determined by SDS Page electrophoresis. As indicated above, epithelial cells associated with ameloblasts are believed to be induced to undergo apoptosis by degradation products migrating from the enamel matrix during dental enamel development. Such degradation products, which generally

have a molecular weight between about 3 kDa and 25 kDa, such as between 5 kDa and 20 kDa, may be particularly effective for use according to the present invention.

The proteins for use according to the invention are normally presented in the form of a preparation, wherein the protein content of the active enamel substance in the preparation is in a range of from about 0.05% w/w to 100% w/w such as, e.g., about 5-99% w/w, about 10-95% w/w, about 15-90% w/w, about 20-90% w/w, about 30-90% w/w, about 40-85% w/w, about 50-80% w/w, about 60-70% w/w, about 70-90% w/w, or about 80-90% w/w.

A preparation of an active enamel substance for use according to the invention may also contain a mixture of proteins with different molecular weights.

The proteins of an enamel matrix can be divided into a high molecular weight part and a low molecular weight part, and it has been found that a well-defined fraction of enamel matrix proteins possesses valuable properties with respect to treatment of periodontal defects (i.e. periodontal wounds). This fraction contains acetic acid extractable proteins generally referred to as amelogenins and constitutes the low molecular weight part of an enamel matrix (cf. EP-B-0 337 967 and EP-B-0 263 086).

As discussed above the low molecular weight part of an enamel matrix has a suitable activity for inducing binding between hard tissues in periodontal defects. In the present context, however, the active proteins are not restricted to the low molecular weight part of an enamel matrix. At present, preferred proteins include enamel matrix proteins such as amelogenin, amelin, tuftelin, etc. with molecular weights (as measured in vitro with SDS-PAGE) below about 60,000 daltons but proteins having a molecular weight above 60,000 daltons have also promising properties as candidates for wound healing, anti-bacterial and/or anti-inflammatory agents.

Accordingly, it is contemplated that the active enamel substance for use according to the invention has a molecular weight of up to about 40,000 such as, e.g. a molecular weight of between about 5,000 and about 25,000.

Within the scope of the present invention are also peptides as described in WO 97/02730, i.e. peptides which comprise at least one sequence element selected from the group con-

sisting of the tetrapeptides DGEA (Asp-Gly-Glu-Ala), VTKG (Val-Thr-Lys-Gly), EKGE (Glu-Lys-Gly-Glu) and DKGE (Asp-Lys-Gly-Glu) and which further comprise an amino acid sequence from which a consecutive string of 20 amino acids is identical to a degree of at least 80% with a string of amino acids having the same length selected from the group consisting of the amino acid sequence shown in SEQ ID NO:1 and a sequence consisting of amino acids 1 to 103 of SEQ ID NO:1 and amino acids 6 to 324 of SEQ ID NO:2 shown in WO 97/02730.

By the term "sequence identity" is meant the identity in sequence of amino acids in the match with respect to identity and position of the amino acids of the peptides. A gap is counted as non-identity for one or more amino acids as appropriate.

Such peptides may comprise from 6 to 300 amino acids, e.g. at least 20 amino acids, at least 30 amino acids, such as at least 60 amino acids, at least 90 amino acids, at least 120 amino acids, at least 150 amino acids or at least 200 amino acids.

A method for the isolation of enamel matrix proteins involves extraction of the proteins and removal of calcium and phosphate ions from solubilized hydroxyapatite by a suitable method, e.g. gel filtration, dialysis or ultrafiltration (see e.g. Janson, J-C & Rydén, L. (Eds.), Protein purification, VCH Publishers 1989 and Harris, ELV & Angal, S., Protein purification methods - A practical approach, IRL Press, Oxford 1990).

A typical lyophilized protein preparation may mainly or exclusively up to 70-90% contain amelogenins with a molecular weight (MW) between 40,000 and 5,000 daltons, the 10-30% being made up of smaller peptides, salts and residual water. The main protein bands are at 20 kDa, 12-14 kDa and around 5 kDa.

By separating the proteins, e.g. by precipitation, ion-exchange chromatography, preparative electrophoresis, gel permeation chromatography, reversed phase chromatography or affinity chromatography, the different molecular weight amelogenins can be purified.

The combination of molecular weight amelogenins may be varied, from a dominating 20 kDa compound to an aggregate of amelogenins with many different molecular weights between 40 and 5 kDa, and to a dominating 5 kDa compound. Other enamel matrix pro-

teins such as amelin, tuftelin or proteolytic enzymes normally found in enamel matrix, can be added and carried by the amelogenin aggregate.

As an alternative source of the enamel matrix derivatives or proteins one may also use generally applicable synthetic routes well-known for a person skilled in the art or use cultivated cells or bacteria modified by recombinant DNA-techniques (see, e.g., Sambrook, J. et al.: Molecular Cloning, Cold Spring Harbor Laboratory Press, 1989).

Physico-chemical properties of enamel matrix, enamel matrix derivatives and enamel matrix proteins

In general the enamel matrix, enamel matrix derivatives and enamel matrix proteins are hydrophobic substances, i.e. less soluble in water especially at increased temperatures. In general, these proteins are soluble at non-physiological pH values and at a low temperature such as about 4-20°C, while they will aggregate and precipitate at body temperature (35-37°C) and neutral pH.

At least a part of the active enamel substance may be in the form of aggregates or is capable of forming aggregates after application in vivo. The particle size of the aggregates is in a range of from about 20 nm to about 1 μm.

It is contemplated that the solubility properties of the active enamel substance are of importance in connection with the prophylactic and therapeutic activity of the substance. When a composition containing the active enamel substance is administered to e.g. a human, the proteinaceous substances will precipitate due to the pH normally prevailing under physiological conditions. Thus, a layer of active enamel substance is formed at the application site and this layer (which also may be a molecular layer in those cases where aggregates have been formed) is difficult to rinse off under physiological conditions. Furthermore, due to the substances bioadhesive properties (see below) the precipitated layer is firmly bound to the tissue also at the margin between the precipitated layer and the tissue. The proteinaceous layer thus covers the tissue onto which the active enamel substance or compositions thereof have been applied and the active enamel substances are maintained in situ for a prolonged period of time, i.e. it is not necessary to administer the active enamel substance(s) with short intervals. Furthermore, the layer formed in situ

can almost be compared to an occlusive dressing, i.e. the layer formed protects the tissue onto which the layer is formed from the surroundings.

In order to enable a proteinaceous layer to be formed in situ after application it may be advantageous to incorporate a suitable buffer substance in a pharmaceutical composition of the active enamel substance; the purpose of such a buffer substance could be to avoid the dissolution of the active enamel substance at the application site.

The active enamel substance has also been observed (by the present inventors) to possess bioadhesive properties, i.e. it has an ability to adhere to skin or mucosal surfaces. These properties are most valuable in connection with a therapeutic and/or prophylactic treatment at least for the following reasons:

- the prophylactically and/or therapeutically active substance(s) can be maintained at the application site for a prolonged period of time (i.e. i) the administration frequency can be reduced, ii) a controlled release effect of the active substance is obtainable and/or iii) a local treatment at the application site is improved)

- the active enamel substance may in itself be suitable as a vehicle for other prophylactically or therapeutically active substances because a vehicle containing the active enamel substance can be formulated as a bioadhesive vehicle (i.e. a novel bioadhesive drug delivery system based on the bioadhesive properties of the active enamel substance).

Pharmaceutical compositions

For the administration to an individual (an animal or a human) the active enamel substance and/or a preparation thereof are preferably formulated into a pharmaceutical composition containing the active enamel substance and, optionally, one or more pharmaceutically acceptable excipients.

The compositions may be in form of, e.g., solid, semi-solid or fluid compositions such as, e.g.,

bioabsorbable patches, drenches, dressings, hydrogel dressings, hydrocolloid dressings, films, foams, sheets, bandages, plasters, delivery devices, implants,

powders, granules, granulates, capsules, agarose or chitosan beads, tablets, pills, pellets, microcapsules, microspheres, nanoparticles,

sprays, aerosols, inhalation devices,

gels, hydrogels, pastes, ointments, creams, soaps, suppositories, vagitories,

solutions, dispersions, suspensions, emulsions, mixtures, lotions, enemas,

kits containing e.g. two separate containers, wherein the first one of the containers contains the active enamel substance e.g. in powder or freeze-dried form optionally admixed with other active drug substance(s) and/or pharmaceutically acceptable excipients and the second container containing a suitable medium intended to be added to the first container before use in order to obtain a ready-to-use composition;

and in other suitable forms such as, e.g., implants or coating of implants or in a form suitable for use in connection with implantation or transplantation.

Compositions for application to the skin or to the mucosa are considered most important in connection with the present invention. Thus, a composition comprising the active enamel substance to be administered may be adapted for administration by any suitable route, for example by topical (dermal), oral, buccal, nasal, aural, rectal or vaginal administration, or by administration to a body cavity such as, e.g., the oral, gastrointestinal, lung or abdominal cavity. Furthermore, a composition may be adapted to administration in connection with surgery, e.g. in connection with excision of tumours or neoplastic tissue or in conjunction with radiation therapy.

As mentioned above, a composition of the active enamel substance may be suitable for use during surgery, e.g. for topical application in the form of a gel, film or dry pellet, or as a rinsing solution or treatment with a paste or cream on tissue or surfaces.

The compositions may be formulated according to conventional pharmaceutical practice, see, e.g., "Remington's Pharmaceutical Sciences" and "Encyclopedia of Pharmaceutical Technology", edited by Swarbrick, J. & J. C. Boylan, Marcel Dekker, Inc., New York, 1988.

A pharmaceutical composition comprising an active enamel substance serves as a drug delivery system. In the present context the term "drug delivery system" denotes a pharmaceutical composition (a pharmaceutical formulation or a dosage form) which upon administration presents the active substance to the body of a human or an animal. Thus, the term "drug delivery system" embraces plain pharmaceutical compositions such as, e.g., creams, ointments, liquids, powders, tablets, etc. as well as more sophisticated formulations such as sprays, plasters, bandages, dressings, devices, etc.

Apart from the active enamel substance, a pharmaceutical composition for use according to the invention may comprise pharmaceutically acceptable excipients.

A pharmaceutically acceptable excipient is a substance which is substantially harmless to the individual to which the composition is to be administered. Such an excipient normally fulfils the requirements given by the national health authorities. Official pharmacopoeias such as e.g. the British Pharmacopoeia, the United States of America Pharmacopoeia and The European Pharmacopoeia set standards for pharmaceutically acceptable excipients.

Whether a pharmaceutically acceptable excipient is suitable for use in a pharmaceutical composition is generally dependent on which kind of dosage form is chosen for use for a particular kind of wound. In the following are given examples of suitable pharmaceutically acceptable excipients for use in different kinds of compositions for use according to the invention.

In the following is given a review on relevant pharmaceutical compositions for use according to the invention. The review is based on the particular route of administration. However, it is appreciated that in those cases where a pharmaceutically acceptable excipient may be employed in different dosage forms or compositions, the application of a particular pharmaceutically acceptable excipient is not limited to a particular dosage form or of a particular function of the excipient.

The choice of pharmaceutically acceptable excipient(s) in a composition for use according to the invention and the optimum concentration thereof cannot generally be predicted and must be determined on the basis of an experimental evaluation of the final composition. However, a person skilled in the art of pharmaceutical formulation can find guidance in e.g., "Remington's Pharmaceutical Sciences", 18th Edition, Mack Publishing Company, Easton, 1990.

Compositions for injection or infusion

For systemic, non-topical administration, the composition comprising the active enamel substance may be in a form suited for systemic injection or infusion and may, as such, be formulated with sterile water or an isotonic saline or glucose solution. The composition may be sterilised by conventional sterilisation techniques which are well known in the art. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilised, the lyophilised preparation being combined with the sterile aqueous solution prior to administration. The composition may contain pharmaceutically acceptable excipients as required to approximate physiological conditions, such as buffering agents, tonicity adjusting agents and the like, for instance sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, etc.

Topical compositions

For application to the mucosa or the skin, the compositions for use according to the invention may contain conventionally non-toxic pharmaceutically acceptable carriers and excipients including microspheres and liposomes.

The compositions for use according to the invention include all kinds of solid, semi-solid and fluid compositions. Compositions of particular relevance are e.g. pastes, ointments, hydrophilic ointments, creams, gels, hydrogels, solutions, emulsions, suspensions, lotions, liniments, shampoos, jellies, soaps, sticks, sprays, powders, films, foams, pads, sponges (e.g. collagen sponges), pads, dressings (such as, e.g., absorbent wound dressings), drenches, bandages, plasters and transdermal delivery systems.

The pharmaceutically acceptable excipients may include solvents, buffering agents, preservatives, humectants, chelating agents, antioxidants, stabilizers, emulsifying agents,

suspending agents, gel-forming agents, ointment bases, penetration enhancers, perfumes, and skin protective agents.

Examples of solvents are e.g. water, alcohols, vegetable or marine oils (e.g. edible oils like almond oil, castor oil, cacao butter, coconut oil, corn oil, cottonseed oil, linseed oil, olive oil, palm oil, peanut oil, poppyseed oil, rapeseed oil, sesame oil, soybean oil, sunflower oil, and teaseed oil), mineral oils, fatty oils, liquid paraffin, polyethylene glycols, propylene glycols, glycerol, liquid polyalkylsiloxanes, and mixtures thereof.

Examples of buffering agents are e.g. citric acid, acetic acid, tartaric acid, lactic acid, hydrogenphosphoric acid, diethylamine etc.

Suitable examples of preservatives for use in compositions are parabens, such as methyl, ethyl, propyl p-hydroxybenzoate, butylparaben, isobutylparaben, isopropylparaben, potassium sorbate, sorbic acid, benzoic acid, methyl benzoate, phenoxyethanol, bronopol, bronidox, MDM hydantoin, iodopropynyl butylcarbamate, EDTA, benzalconium chloride, and benzylalcohol, or mixtures of preservatives.

Examples of humectants are glycerin, propylene glycol, sorbitol, lactic acid, urea, and mixtures thereof.

Examples of chelating agents are sodium EDTA and citric acid.

Examples of antioxidants are butylated hydroxy anisole (BHA), ascorbic acid and derivatives thereof, tocopherol and derivatives thereof, cysteine, and mixtures thereof.

Examples of emulsifying agents are naturally occurring gums, e.g. gum acacia or gum tragacanth; naturally occurring phosphatides, e.g. soybean lecithin; sorbitan monooleate derivatives; wool fats; wool alcohols; sorbitan esters; monoglycerides; fatty alcohols; fatty acid esters (e.g. triglycerides of fatty acids); and mixtures thereof.

Examples of suspending agents are e.g. celluloses and cellulose derivatives such as, e.g., carboxymethyl cellulose, hydroxyethylcellulose, hydroxypropylcellulose, hydroxypropylmethylcellulose, carraghenan, acacia gum, arabic gum, tragacanth, and mixtures thereof.

Examples of gel bases, viscosity-increasing agents or components which are able to take up exudate from a wound are: liquid paraffin, polyethylene, fatty oils, colloidal silica or aluminium, zinc soaps, glycerol, propylene glycol, tragacanth, carboxyvinyl polymers, magnesium-aluminium silicates, Carbopol®, hydrophilic polymers such as, e.g. starch or cellulose derivatives such as, e.g., carboxymethylcellulose, hydroxyethylcellulose and other cellulose derivatives, water-swellable hydrocolloids, carragenans, hyaluronates (e.g. hyaluronate gel optionally containing sodium chloride), and alginates including propylene glycol aginate.

Examples of ointment bases are e.g. beeswax, paraffin, cetanol, cetyl palmitate, vegetable oils, sorbitan esters of fatty acids (Span), polyethylene glycols, and condensation products between sorbitan esters of fatty acids and ethylene oxide, e.g. polyoxyethylene sorbitan monooleate (Tween).

Examples of hydrophobic or water-emulsifying ointment bases are paraffins, vegetable oils, animal fats, synthetic glycerides, waxes, lanolin, and liquid polyalkylsiloxanes.

Examples of hydrophilic ointment bases are solid macrogols (polyethylene glycols).

Other examples of ointment bases are triethanolamine soaps, sulphated fatty alcohol and polysorbates.

Examples of powder components are: alginate, collagen, lactose, powder which is able to form a gel when applied to a surgical wound (absorbs liquid/wound exudate). Normally, powders intended for application on large open wounds must be sterile and the particles present must be micronized.

Examples of other excipients are polymers such as carmelose, sodium carmelose, hydroxypropylmethylcellulose, hydroxyethylcellulose, hydroxypropylcellulose, pectin, xanthan gum, locust bean gum, acacia gum, gelatin, carbomer, emulsifiers like vitamin E, glyceryl stearates, cetanyl glucoside, collagen, carrageenan, hyaluronates and alginates and chitosans.

Dressings and/or bandages are also important delivery systems for the active enamel substance. When dressings are used as dosage form, the active enamel substance may be admixed with the other ingredients before or during the manufacture of the dressing or the active enamel substance may in some way be coated onto the dressing e.g. by dipping the dressing in a solution or dispersion of the active enamel substance or by spraying a solution or dispersion of the active enamel substance onto the dressing. Alternatively, the active enamel substance may be applied in the form of a powder to the dressing. Dressings may be in the form of absorbent wound dressings for application to exuding wounds. Dressings may also be in the form of hydrogel dressings (e.g. cross-linked polymers such as, e.g. Intrasite® which contains carboxymethylcellulose, propylene glycol or polysaccharide, disaccharide and proteins) or in the form of occlusive dressings such as, e.g., alginates, chitosan, hydrophilic polyurethane film, collagen sheets, plates, powders, foams, or sponges, foams (e.g. polyurethane or silicone), hydrocolloids (e.g. carboxymethylcellulose, CMC), collagen and hyaluronic acid-based dressings including combinations thereof.

The compositions mentioned above for topical administration are most suitably for application directly to wounds or they may be suitable for application to or for introduction into relevant orifice(s) of the body, e.g. the rectal, urethral, vaginal, aural, nasal or oral orifices. The composition may simply be applied directly on the part to be treated such as, e.g., on the mucosa, or by any convenient route of administration.

Compositions which have proved to be of importance in connection with topical application are those which have thixotropic properties, i.e. the viscosity of the composition is affected e.g. by shaking or stirring so that the viscosity of the composition at the time of administration can be reduced and when the composition has been applied, the viscosity increases so that the composition remains at the application site.

Compositions for application to mucosa or skin

Suitable compositions for use according to the invention may also be presented in the form of suspensions, emulsions or dispersions. Such compositions contains the active enamel substance in admixture with a dispersing or wetting agent, suspending agent, and/or one or more preservatives and other pharmaceutically acceptable excipients. Such compositions may also be suitable for use in the delivery of the active enamel substance

to e.g. an intact or damaged mucosa such as the oral, buccal, nasal, rectal, or vaginal mucosa, or for administration to intact or damaged skin, or wounds.

Suitable dispersing or wetting agents are, for example, naturally occurring phosphatides, e.g., lecithin, or soybean lecithin; condensation products of ethylene oxide with e.g. a fatty acid, a long chain aliphatic alcohol, or a partial ester derived from fatty acids and a hexitol or a hexitol anhydride, for example polyoxyethylene stearate, polyoxyethylene sorbitol monooleate, polyoxyethylene sorbitan monooleate, etc.

Suitable suspending agents are, e.g., naturally occurring gums such as, e.g., gum acacia, xanthan gum, or gum tragacanth; celluloses such as, e.g., sodium carboxymethylcellulose, microcrystalline cellulose (e.g. Avicel® RC 591, methylcellulose); alginates and chitosans such as, e.g., sodium alginate, etc.

Suitable examples of preservatives for use in compositions according to the invention are the same as those mentioned above.

Rectal and/or vaginal compositions

For application to the rectal or vaginal mucosa, suitable compositions according to the invention include suppositories (emulsion or suspension type), enemas, and rectal gelatin capsules (solutions or suspensions). Appropriate pharmaceutically acceptable suppository bases include cocoa butter, esterified fatty acids, glycerinated gelatin, and various water-soluble or dispersible bases like polyethylene glycols and polyoxyethylene sorbitan fatty acid esters. Various additives like, e.g., enhancers or surfactants may be incorporated.

Nasal or pulmonary compositions

For application to the nasal or pulmonal mucosa (as well as to the oral mucosa), sprays and aerosols for inhalation are suitable compositions according to the invention. In a typical composition, the active enamel substance is present in the form of a particulate formulation optionally dispersed in a suitable vehicle. The pharmaceutically acceptable vehicles and excipients and optionally other pharmaceutically acceptable materials present in the composition such as diluents, enhancers, flavouring agents, preservatives, etc.

are all selected in accordance with conventional pharmaceutical practice in a manner understood by the persons skilled in the art of formulating pharmaceuticals.

Dosages of enamel matrix, enamel matrix derivatives and enamel matrix proteins

In a pharmaceutical composition for use according to the invention, an active enamel substance is generally present in a concentration ranging from about 0.01% to about 99.9% w/w. The amount of composition applied will normally result in an amount of total protein per cm^2 area of affected tissue corresponding to from about 0.01 mg/cm^2 to about 20 mg/cm^2 such as from about 0.1 mg/cm^2 to about 15 mg/cm^2.

The amount applied of the composition depends on the concentration of the active enamel substance in the composition and of the release rate of the active enamel substance from the composition, but is generally in a range corresponding to at the most about 15-20 mg/cm^2.

In those cases where the active enamel substance is administered in the form of a liquid composition, the concentration of the active enamel substance in the composition is in a range corresponding to from about 0.1 to about 50 mg/ml. Higher concentrations are in some cases desirable and can also be obtained such as a concentration of at least about 100 mg/ml.

The concentration of the active enamel substance in a pharmaceutical composition depends on the specific enamel substance, its potency, the severity of the disease to be prevented or treated, and the age and condition of the patient. Methods applicable to selecting relevant concentrations of the active enamel substance in the pharmaceutical composition are well known to a person skilled in the art and may be performed according to established guidelines for good clinical practice (GCP) or Investigational New Drug Exemption ("IND") regulations as described in e.g. International Standard ISO/DIS 14155 Clinical investigation of medical devices, 1994 and ICH (International Committee for Harmonisation): Harmonised tripartite guideline for good clinical practice, Brookwood Medical Publications, Ltd, Surrey, UK, 1996. A person skilled in the art would, by use of the methods described in standard textbooks, guidelines and regulations as described above as well as common general knowledge within the field, be able to select the exact dosage

regimen to be implemented for any active enamel substance and/or selected other active substances and dosage form using merely routine experimentation procedures.

In accordance with the present invention, application of the active enamel substance at or on tumorous tissue may suitably be combined with other forms of tumour treatment, such as surgery, administration of chemotherapeutic agents and/or radiation therapy of affected tissue.

The invention is further described in the following examples which are not in any way intended to limit the scope of the invention as claimed.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is further described in the following with reference to the appended drawings, wherein

Fig. 1 is a graph showing the density of human epithelial (HeLa) cells grown in the presence and absence of EMD;

Fig. 2 is a graph showing the production of intracellular cAMP of HeLa cells grown in the presence or absence of EMD; and

Fig. 3 is a graph showing induced cell death of HeLa cells grown in the presence of EMD compared to HeLa cells grown in the absence of EMD as measured by the level of apoptosis specific nucleic acid degradation products.

The present invention is further described in the following examples which are not in any way intended to limit the scope of the invention as claimed.

EXPERIMENTAL SECTION

Enamel Matrix Derivative, EMDOGAIN®, from BIORA AB, S-205 12 Malmö, Sweden containing 30 mg freeze-dried enamel matrix protein (in the following abbreviated to EMD) and 1 ml vehicle solution (Propylene Glycol Alginate), which are mixed prior to application,

unless the protein and the vehicle are tested separately. The weight ratio is about 85/5/10 between the main protein peaks at 20, 14 and 5 kDa, respectively.

Example 1

Apoptosis in guinea pig dental epithelium

Tissue preparation

Two guinea pigs of 200-250 g were anesthetized by carbon dioxide and decapitated. Bilateral maxilla and mandible were dissected and fixed with freshly prepared 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4, at 4°C. After fixation, the specimens were decalcified with neutral buffered 10% EDTA, dehydrated with graded ethanol, and embedded in paraffin. Sections (7 μm) were taken in the following directions: sagittal (mesio-distal) sections to obtain information about the changes in the enamel epithelium in association with the formation of the cementum pearls that are formed on the enamel surface, bucco-lingual sections to obtain information about changes in the epithelial root sheath that take place when dentin and cementum are formed at the apical end of the molars, horizontal sections to follow the distribution of the epithelium around the molars. These sections were used for immunobistochemical demonstration of the epithelial cells and their basal lamina as well as the possible occurrence of apoptosis. Some sections were stained with hematoxylin and eosin (H&E).

Immunohistochemical detection of laminin and keratin

The sections were pretreated with 2% hydrogen peroxide to diminish endogenous peroxidase, incubated with 0.1% protease (Sigma, St,Louis, USA) for 15 min, and non-specific reaction was blocked with 4% normal goat serum (Dako, Copenhagen, Denmark). Then, they were incubated with rabbit anti-laminin antibody (1:1000, Dako, Copenhagen, Denmark) or mouse monoclonal anti-cytokeratin antibody (1:100, Boehringer-Mannheim, Germany) at 4°C overnight, rinsed with TBS (50 mM Tris buffer saline, pH 7.4), incubated with goat anti-rabbit antibody with HRP (Dako, Copenhagen, Denmark) or goat anti-mouse antibody with HRP diluted to 1:100 (Dako, Copenhagen, Denmark) at room temperature for 60 min. The immunoreaction was visualized with 0.1% DAB (diamino benzi-

dine), TBS were substituted for the primary antibodies in controls*TUNEL procedure for the visualization of apoptosis*

The sections were deparaffinized with xylene, rehydrated with graded ethanol, and rinsed with PBS (phosphate-buffered saline, 50 mM sodium phosphate, 200 mM NaCl, pH 7.4). The sections were incubated with proteinase K (20 μg/ml in PBS, pH 7.4, Sigma, St.Louis, USA) for 15 min. at 37°C to expose the DNA strands, and rinsed with PBS. Endogenous peroxidase was blocked by 2.0% hydrogen peroxide in PBS for 5 min. at room temperature, DIG labeled dUTP (deoxyuridine triphosphate) solution and TdT (terminal deoxynucleotidyl transferase) were mixed to make TUNEL mixture (Oncor, Gaithersburg, MD). The sections were incubated with TUNEL mixture for 60 min. at 37°C. DIG labeled dUTP solution without TdT (terminal deoxynucleotidyl transferase) was used as negative control. After incubation, the specimens were rinsed with PB S and reacted with anti-Digoxigenin-peroxidase for 30 min. at room temperature. The reaction was visualized by 0.1% DAB with 0-02% hydrogen peroxide in PBS at room temperature. For positive controls, a mandible and a spleen of rats was observed in the same manner. Apoptotic cells were distributed in a part of incisal ameloblasts of transition-stage and a part of disintegrated enamel epithelium from FIRS in rat molars. Apoptotic cells were scattered in the spleen of a rat.

Results

The immunohistochemistry with antibodies against keratin showed the epithelial cells in all positions where they could be identified by means of ordinary light microscopy. In addition it was possible to distinguish epithelial cells in areas where cells of the enamel organ or the epithelial root sheath were mingled with mesenchymal cells of the dental follicle. Immunohistochemistry with antibodies against laminin showed that the epithelial structures were associated with a basement membrane in some areas and that a basement membrane was missing in others. The TUNEL method visualized apoptotic bodies in specific regions of the contiguously growing teeth.

It was observed that ameloblasts underwent apoptosis in early secretory stage, transition stage, maturation stage and reduced enamel epithelial stage. Apoptosis in maturation stage and reduced enamel epithelial stage appeared to be associated with the formation of cementum pearls. At the apical area of the cartilage-like cementum, the enamel organ which had a large stellate reticulum and lacked the distinct border of outer enamel epithe-

lium was observed. Apoptosis of enamel epithelium was observed in the same portions as those of cementum pearls. Based on these results, it is considered likely that apoptosis plays an important part in the reduction, transformation, evacuation and migration of enamel epithelium, which is an important step for the formation of cementum during tooth development.

Example 2

Growth of human epithelial cells in the presence and absence of EMD

Materials and Methods

Human epithelial cells (HeLa; a human cervical cancer cell line) were obtained from BioWhittaker, HeLa 07-229c, Lot # 8c2720. The cells were grown in Modified Eagle's Medium supplemented with 10% fetal calf serum. EMD was supplied by surface coating culture dishes with a 0.1% EMD solution in 0.1% HAc, and by supplementing the culture medium with 100 μg EMD per ml of medium. HeLa cells cultured under similar conditions in the absence of EMD were used as controls. All experiments were initiated with 50,000 cells per ml of culture medium.

Results

(a) HeLa cells were grown in cultures for 24, 48, 72, 96 and 120 hours. Cultures were then washed with PBS and cells were counted in the microscope using a fixed grid. Five different areas were counted in each of six parallel cultures at each timepoint. As appears from Fig. 1, HeLa cells show a marked decrease in cell density from 48 hours when grown in the presence of EMD.

(b) HeLa cells were cultured for 24 or 120 hours, washed twice with PBS and centrifuged. 100 μl of cells from each culture (n=6 at each timepoint/experiment) were then lysed, and released intracellular cAMP was measured by competitive enzyme immunoassay (EIA) using an Amersham Pharmacia Biotech "Biotrak cAMP EIA" kit (Cat. No. RPN 225) in accordance with the manufacturer's instructions. Compared to controls, HeLa cells show a marked increase in intracellular cAMP after 24 hours of growth in the presence of EMD (Fig. 2). This increase could still be observed after 120 hours in culture. The increase in

intracellular cAMP suggests that cells grown in the presence of EMD generate internal signal(s) that could be part of pathways for growth regulation and differentiation.

(c) HeLa cells were harvested from cultures at 24, 48, 72, 96 or 120 hours (n=5 at each timepoint/experiment), washed in PBS and centrifuged. 200 µl of cells were lysed, and the level of apoptosis specific nucleic acid degradation products (histone associated DNA fragments) was quantified by sandwich ELISA using a Boehringer Mannheim "Cell Death Detection ELISA" kit (Cat. No. 1 774 425) according to the manufacturer's instructions. The results are presented as the ratio between EMD treated cells and untreated cells. Hence values above 1 indicate induced cell death while values below 1 reflect prolonged cell survival. It appears from Fig. 3 that the HeLa cells show a marked increase in induced cell death when EMD is present in the cultures (values above 1), peaking at 72 hours after addition of EMD.

Based on these results, it is concluded that epithelial cell growth is poorer in the presence of EMD, and that the presence of EMD in the cultures increased programmed cell death more than two-fold.

Example 3

Growth of human cancer cells in the presence of EMD

Materials and Methods

Human cancer cells were obtained from cell culture banks derived from tumour tissues from patients undergoing cancer treatment at the Norwegian Cancer Hospital in Oslo, Norway. The cells were grown in Dulbecco's Modiefied Eagle's Medium supplemented with 10% fetal calf serum (osteosarcoma cells) or Eagle's Modified Eagle's Medium supplemented with 10% fetal calf serum (epithelial derived cells). EMD was supplied by surface coating culture dishes with a 0.5 mg/ml EMD solution in 0.01% HAc, and by supplementing the culture medium with 100 µg EMD per ml of medium. All experiments were initiated with 50,000 cells per ml of culture medium.

Results

Cells were harvested from the cultures at 72 and 120 hours (n=3x3 each time). The cells were washed in PBS and centrifuged, and 200µl of cells from each sample were lysed and the level of apoptosis specific nucleic acid degradation products (histone associated DNA fragments) was quantified by sandwich ELISA using a Boehringer Mannheim "Cell Death Detection ELISA" kit (Cat. No. 1 774 425) according to the manufacturer's instructions. The results are presented as the ratio between EMD treated cells and untreated cells. Hence values above 1 indicate induced cell death while values below 1 reflect prolonged cell survival.

It appears from Table 1 below that human cancer cells show a marked increase (values above 1) in induced cell death in the presence of EMD in the cell cultures, peaking at 72 to 120 hours after addition of EMD.

Table 1

Tissue of origin cell line	**72 hours after addition of EMD**	**120 Hours after addition of EMD**
Mammary glands		
MCF-7	1.1	1.3
SK-BR-3	1.1	1.2
T47D	1.3	1.2
ZR35	2.1	1.8
Osteosarcoma (bone)		
OHS	2.5	1.4
Melanoma (skin)		
LOX	1.1	1.1
FEMX-1	1.5	1.4
Ovarian carcinoma		
OVCAR	3.5	2.5
SK-OV-3	2.8	1.4
Rhabdomyosarcoma (muscle)		
RH-28	2.7	1.7

CLAIMS

1. Use of a preparation of an active enamel substance for the preparation of a pharmaceutical composition for the induction of apoptosis.

2. Use of a preparation of an active enamel substance for the preparation of a pharmaceutical composition for the prevention or treatment of malignant or benign neoplasms.

3. Use of a preparation of an active enamel substance for the preparation of a pharmaceutical composition for the prevention or treatment of cancer.

4. Use of a preparation of an active enamel substance for the preparation of a pharmaceutical composition for the (selective) induction of apoptosis in neoplastic cells.

5. Use according to any of claims 1-4 wherein the preparation of active enamel substance is applied at or on tissue comprising a substantial proportion of epithelial cells.

6. Use according to claim 5 wherein said tissue comprises skin or mucosal tissue.

7. Use according to claim 6 wherein the mucosal tissue comprises oral mucosa, gastrointestinal mucosa, mucosa of the respiratory tract (such as lung mucosa), cervical mucosa or abdominal mucosa.

8. Use according to claim 5 wherein the tissue is glandular tissue, e.g. mammary gland, pancreas, liver, thyroid gland, bladder, prostate, sweat gland, salivary gland or pituitary gland tissue.

9. Use according to any of claims 1-4 for the topical treatment of cancer or neoplasms.

10. Use according to claim 8 wherein the cancer or neoplasm is selected from epithelially derived cancers or neoplasms.

11. Use according to claim 9 for application at or on a tumour site before, during or after a tumour operation to substantially reduce the risk of post-surgical metastasis or to substantially prevent recurrence of the tumour.

12. Use according to any of the preceding claims, wherein the active enamel substance is enamel matrix, enamel matrix derivatives and/or enamel matrix proteins.

13. Use according to any of the preceding claims, wherein the active enamel substance is selected from the group consisting of enamelins, amelogenins, non-amelogenins, proline-rich non-amelogenins, amelins (ameloblastin, sheathlin), tuftelins, and derivatives thereof and mixtures thereof.

14. Use according to any of the preceding claims, wherein the active enamel substance has a molecular weight of at the most about 120 kDa such as, e.g, at the most 100 kDa, 90 kDa, 80 kDa, 70 kDa or 60 kDa as determined by SDS Page electrophoresis.

15. Use according to any of the preceding claims, wherein the preparation of an active enamel substance contains a mixture of active enamel substances with different molecular weights.

16. Use according to any of the preceding claims, wherein the preparation of an active enamel substance comprises at least two substances selected from the group consisting of amelogenins, proline-rich non-amelogenins, enamelins, tuftelin, tuft proteins, serum proteins, salivary proteins, amelin, ameloblastin, sheathlin, and derivatives thereof.

17. Use according to any of the preceding claims, wherein the active enamel substance has a molecular weight of up to about 40,000.

18. Use according to any of the preceding claims, wherein the active enamel substance has a molecular weight of between about 5,000 and about 25,000.

19. Use according to any of the preceding claims, wherein the major part of the active enamel substance has a molecular weight of about 20 kDa.

20. Use according to any of the preceding claims, wherein at least a part of the active enamel substance is in the form of aggregates or after application in vivo is capable of forming aggregates.

21. Use according to claim 20, wherein the aggregates have a particle size of from about 20 nm to about 1 μm.

22. Use according to any of the preceding claims, wherein the protein content of the active enamel substance in the preparation is in a range of from about 0.05% w/w to 100% w/w such as, e.g., about 5-99% w/w, about 10-95% w/w, about 15-90% w/w, about 20-90% w/w, about 30-90% w/w, about 40-85% w/w, about 50-80% w/w, about 60-70% w/w, about 70-90% w/w, or about 80-90% w/w.

23. Use according to any of the preceding claims, wherein the pharmaceutical composition further comprises a pharmaceutically acceptable excipient.

24. Use according to claim 23, wherein the pharmaceutically acceptable excipient is propylene glycol alginate.

25. Use according to claim 23, wherein the pharmaceutically acceptable excipient is hyaluronic acid or salts or derivatives thereof.

26. Use according to any of claims 1-25 of EMDOGAIN® or any proteins or peptides contained therein for the induction of apoptosis.

27. A method for inducing of apoptosis in neoplastic cells, the method comprising applying an effective amount of an active enamel substance at or on neoplastic cells.

28. A method for preventing or treating malignant or benign neoplasms, the method comprising administering to a mammal in need thereof a prophylactically or therapeutically effective amount of an active enamel substance.

29. A method according to claim 28, wherein the active enamel substance is applied in an amount of total protein per cm^2 area of affected tissue corresponding to from about 0.01 mg/cm^2 to about 20 mg/cm^2, such as from about 0.1 mg/cm^2 to about 15 mg/cm^2.

ABSTRACT

Matrix Protein Compositions for Induction of Apoptosis

Enamel matrix, enamel matrix derivatives and/or enamel matrix proteins or peptides may be used as therapeutic or prophylactic agents for inducing programmed cell death (apoptosis), in particular in the treatment or prevention of cancer or malignant or benign neoplasms.

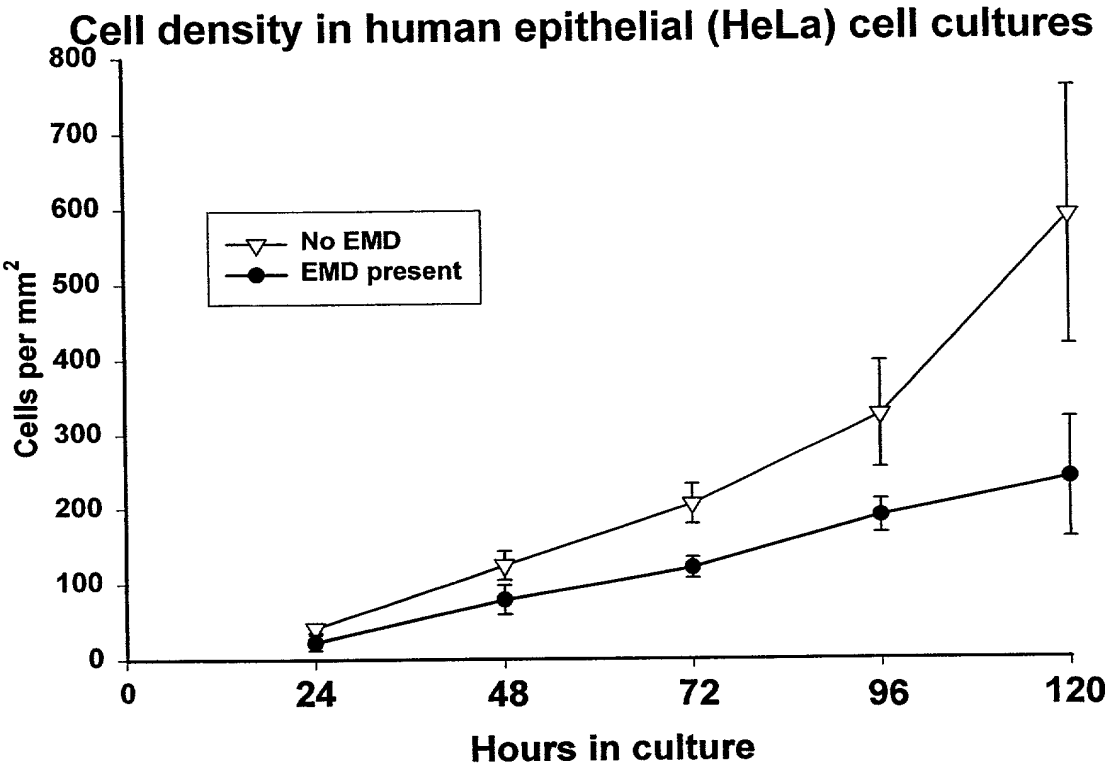

Cell density in human epithelial (HeLa) cell cultures
800
700
600
500
400
300
200
100
0
Cells per mm2
No EMD
EMD present
0
24
48
72
96
120
Hours in culture

Fig. 1

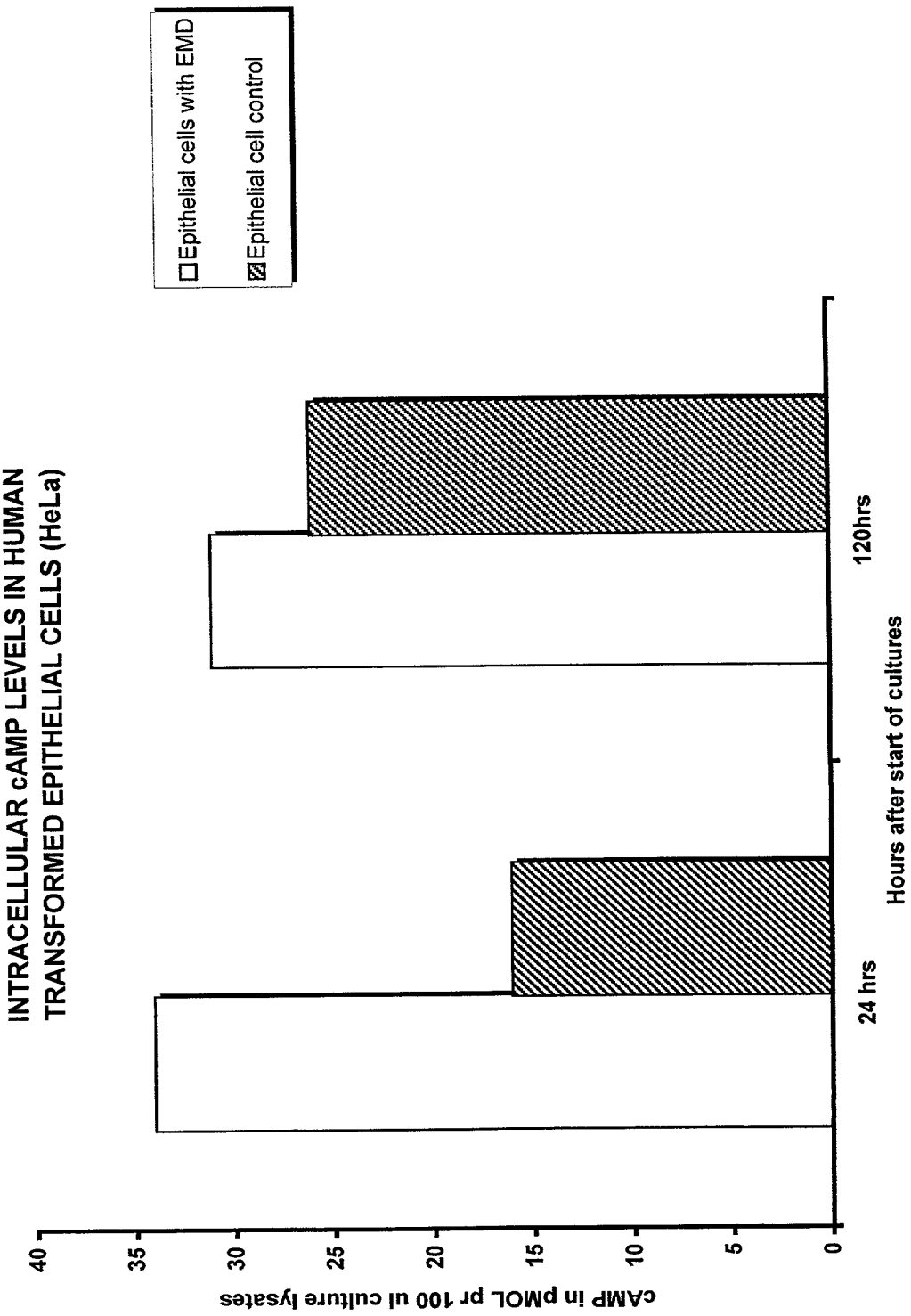

INTRACELLULAR cAMP LEVELS IN HUMAN TRANSFORMED EPITHELIAL CELLS (HeLa)
Epithelial cells with EMD
Epithelial cell control
cAMP in pMOL pr 100 ul culture lysates
0
5
10
15
20
25
30
35
40
24 hrs
120hrs
Hours after start of cultures

Fig. 2

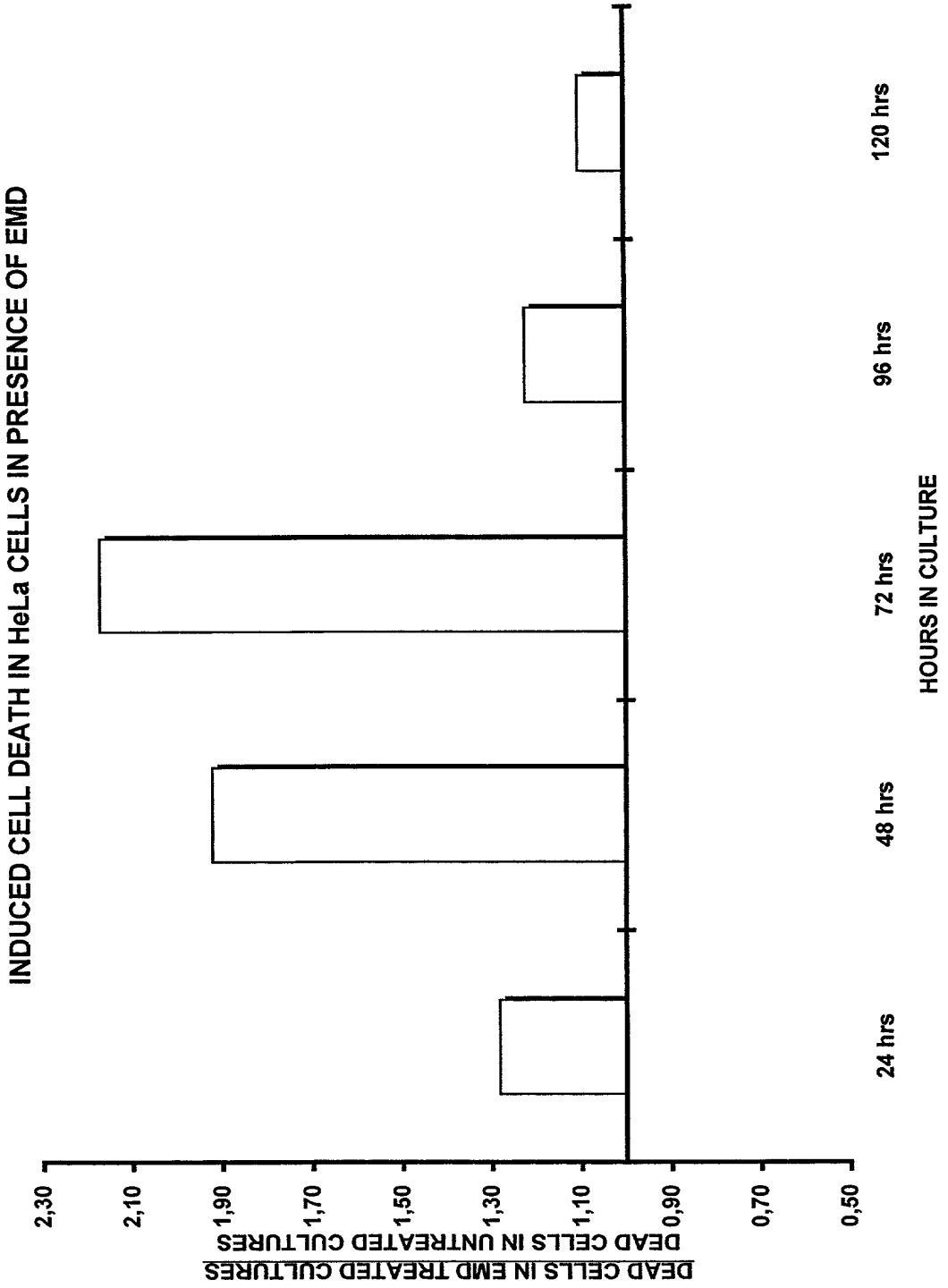

INDUCED CELL DEATH IN HeLa CELLS IN PRESENCE OF EMD
DEAD CELLS IN EMD TREATED CULTURES
DEAD CELLS IN UNTREATED CULTURES
2,30
2,10
1,90
1,70
1,50
1,30
1,10
0,90
0,70
0,50
24 hrs
48 hrs
72 hrs
96 hrs
120 hrs
HOURS IN CULTURE

Fig. 3

Docket No.
49122

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

MATRIX PROTEIN COMPOSITIONS FOR INDUCTION OF APOPTOSIS

the specification of which

(check one)

☒ is attached hereto.

☐ was filed on _____ as United States Application No. or PCT International Application Number _____

and was amended on _____

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
DK PA 1999 00336	Denmark	10 March 1999	☐
(Number)	(Country)	(Day/Month/Year Filed)	
			☐
(Number)	(Country)	(Day/Month/Year Filed)	
			☐
(Number)	(Country)	(Day/Month/Year Filed)	

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

60/134,813	May 19, 1999
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

Peter F. Corless (Reg. No. 33,860)
Sewall P. Bronstein (Reg. No. 16,919)
David G. Conlin (Reg. No. 27,026)
George W. Neuner (Reg. No. 26,964)
Linda M. Buckley (Reg. No. 31,003)
Peter J. Manus (Reg. No. 26,766)
Cara Z. Lowen (Reg. No. 38,227)
William J. Daley, Jr. (Reg. No. 35,487)
Robert L. Buchanan (Reg. No. 40,927)
Christine C. O'Day (Reg. No. 38,256)
Richard Gamache (Reg. No. 39196)

Send Correspondence to: **Peter F. Corless**
Dike, Bronstein, Roberts & Cushman, LLP
130 Water Street
Boston, MA 02109

Direct Telephone Calls to: *(name and telephone number)*
Peter F. Corless (617) 523-3400

Full name of sole or first inventor
Lars HAMMARSTRÖM

Sole or first inventor's signature — Date
Lars Hammarström — 3 March 2000

Residence
Frejavägen 28, S-182 64 Djursholm, Sweden

Citizenship
Swedish

Post Office Address
Same

Full name of second inventor, if any
Ståle Petter LYNGSTADAAS

Second inventor's signature — Date
6. MARCH 2000

Residence
Haakonsvei 5, N-1450 Nesoddtangen, Norway

Citizenship
Norwegian

Post Office Address
Same

Full name of third inventor, if any
Stina GESTRELIUS
Third inventor's signature — Date: 1 March 2000
Residence: St. Sigridsg. 5, S-223 50 Lund, Sweden
Citizenship: Swedish
Post Office Address: Same

Full name of fourth inventor, if any
Fourth inventor's signature — Date
Residence
Citizenship
Post Office Address

Full name of fifth inventor, if any
Fifth inventor's signature — Date
Residence
Citizenship
Post Office Address

Full name of sixth inventor, if any
Sixth inventor's signature — Date
Residence
Citizenship
Post Office Address